PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A61K 37/02, C12N 5/08
C12Q 1/00

A1

(11) International Publication Number: WO 93/19770

(43) International Publication Date: 14 October 1993 (14.10.93)

(21) International Application Number: PCT/GB93/00719

(22) International Filing Date: 6 April 1993 (06.04.93)

(30) Priority data:
9207732.0 6 April 1992 (06.04.92) GB

(71) Applicant *(for all designated States except US)*: ISIS INNOVATION LIMITED [GB/GB]; 2 South Parks Road, Oxford 0X1 3UB (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : GORDON, Siamon [ZA/GB]; 6A Canterbury Road, Oxford OX2 6LU (GB). STEIN, Michael [ZA/GB]; 7 Hartley Court, St Margaret's Road, Oxford 0X2 6RU (GB).

(74) Agent: PENNANT, Pyers; Stevens, Hewlett & Perkins, 1 Serjeants' Inn, Fleet Street, London EC4Y 1LL (GB).

(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: COMPOSITIONS FOR THE TREATMENT OF ACUTE OR CHRONIC INFLAMMATION

(57) Abstract

A composition for the treatment of acute or chronic inflammation, comprising IL-4 and IL-10, or Th-2 cell supernatant, r supernatant of macrophages with a healing phenotype, and a corresponding method of treatment. Macrophages with a healing phenotype are a newly defined group of macrophages, derived from elicited macrophages stimulated with IL-4 and IL-10, or Th-2 cell supernatant, and having a clearly distinguishable phenotype.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**FR**	France	**MR**	Mauritania
AU	Australia	**GA**	Gabon	**MW**	Malawi
BB	Barbados	**GB**	United Kingdom	**NL**	Netherlands
BE	Belgium	**GN**	Guinea	**NO**	Norway
BF	Burkina Faso	**GR**	Greece	**NZ**	New Zealand
BG	Bulgaria	**HU**	Hungary	**PL**	Poland
BJ	Benin	**IE**	Ireland	**PT**	Portugal
BR	Brazil	**IT**	Italy	**RO**	Romania
CA	Canada	**JP**	Japan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SK**	Slovak Republic
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SN**	Senegal
CM	Cameroon	**LI**	Liechtenstein	**SU**	Soviet Union
CS	Czechoslovakia	**LK**	Sri Lanka	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**MC**	Monaco	**UA**	Ukraine
DK	Denmark	**MG**	Madagascar	**US**	United States of America
ES	Spain	**ML**	Mali	**VN**	Viet Nam
FI	Finland	**MN**	Mongolia		

COMPOSITIONS FOR THE TREATMENT OF ACUTE OR CHRONIC INFLAMMATION

The mononuclear phagocyte system (MPS) has a central role in innate immunity, would healing and tissue homeostasis and, in addition, is intimately involved in the development and expression of specific immune responses. The MPS is comprised of widely distributed tissue macrophages, circulating monocytes and, in inflamed tissues, recruited macrophages. Bone-marrow derived monocytes, the immature precursors of the MPS, are highly adaptable cells and acquire specialised properties within local and distinct tissue environments. The regulation of the macrophage's diverse functional repertoire, with its extraordinary secretory and endocytic potential, is of significant immunologic interest.

Tissue injury sets in motion a cascade of events designed to eliminate infectious agents and to maximise early reconstitution of the tissues. Among the earliest cells recruited to sites of tissue injury are neutrophils and macrophages. Monocytes are rapidly directed from the circulation to the inflammatory site. Macrophages elicited under such conditions (known as elicited or exudate macrophages), are primed for a role in inflammation with altered expression of receptors and secretory products, but have not acquired maximal microbicidal activity. Subsequent exposure of elicited macrophages to "activating" T lymphocyte-derived cytokines, expressed at the site of inflammation, is crucial to the development of fully microbicidal macrophages. The ability to generate more effective cytocidal mechanisms as well as altered expression of specific endocytic receptors distinguish lymphokine activated cells from those cells with an elicited phenotype.

Foreign particles, for example bacteria, that gain entry into the tissues are rapidly ingested by phagocytic cells, largely resid nt marcrophages. Two major events may ensue; (1) the production of inflammatory mediators leading to recruitment of immune cells, including neutrophils and monocytes, and (2) the processing of ingested material for possible presentation to antigen-specific T-lymphocytes. In most cases, tissue cells or early elicited neutrophils and macrophages kill and degrade the invading microorganisms and the acute inflammatory stimulus is therefore removed. However, in the presence of persistent infection, continued turnover, recruitment and stimulation of mononuclear cells occur. The result is chronic inflammatory infiltrate consisting predominantly of macrophages and T-cells each secreting a range of inflammatory mediators which amplify and perpetuate the lesion. Macrophage products contribute to lymphocyte accumulation and activation by a process of antigen presentation, while lymphocyte products regulate macrophage recruitment and function (Blanden, 1976).

Activated T-helper cells at the inflammatory site and within stimulated lymphoid organs produce a large number of soluble immunoregulatory substances, many of which may have profound influence on macrophage antigen presenting function as well as effector function. Lymphokines act on newly recruited monocytes/macrophages to enhance their host-defense function, a crucial event for effective elimination of many pathogens.

In the quest for the mediator/s of "macrophage activation", collectively termed macrophage activating factor/s (MAF), a variety of

assays, in particular assays of respiratory burst activity, were used to measure the effect of cellular supernatants on human or mouse macrophage populations (Simon and Sheagren, 1972). The major MAF of conditioned media from antigen stimulated T-cells isolated from BCG, Listeria monocytogenes or Leishmania donovani infected mice, was identified as gamma interferon, IFN-g (Nathan et al, 1983; Murray et al, 1983a-b). As blocking mAbs (monoclonal antibodies) and pure protein became available it was demonstrated that IFN-g accounted for many features of the activated macrophage phenotype by "conventional" criteria including enhanced MHC class 2 antigen (Ag)expression, priming for subsequent triggering of the respiratory burst, and enhanced microbicidal activity. It was also demonstrated that IFN-g was capable of inducing macrophage tumoricidal activity (Schreiber et al, 1986).

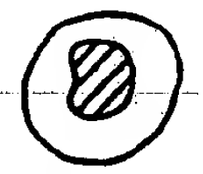
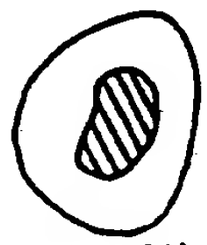
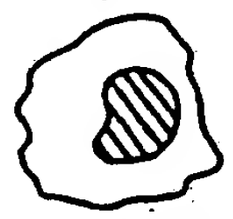
It has been shown that other cytokines have direct or synergistic macrophage activating properties. In humans, one other cytokine, GM-CSF (granulocyte-macrophage colony stimulating factor), stands out as being a particularly potent activator of monocytes, (Reed et al, 1987). Generally however, few cytokines can be regarded as potent and direct macrophage immunologic activators although some may be important "accessory" molecules.

The process of immunologic activation is complex, involving the expression and regulation of multiple gene products. The use of killing mechanisms is only partly useful in defining the activated macrophage phenotype, and more simple antigen-based assays that reflect this process are required for in situ studies of immunologic activation.

Table 1 below shows ways in which immunologically activated cells are distinguished from resting cells (circulating monocytes or tissue macrophages) or elicited cells (attracted to an inflammatory focus by non-specific stimuli), by altered expression of specific markers.

TABLE 1

Markers of Mϕ activation

Secretory products and activities				Plasma membrane markers			
Plasminogen Activator	ROI	Microbicidal Activity		Ia	MMR	CR3	FcR-I
+/-	+	+/-	 Monocyte	+/-	-	+ +	+
+ +	+	+	 elicited Mϕ	+	+	+ +	+ +
+ +	+ +	+ +	 activated Mϕ	+ +	-	+ +	++

Of these, assays of respiratory burst, MHC class 2 Ag expression and MMR (macrophage mannose receptor) are well established as indicators of "conventional" immunologic activation. Other as yet uncharacterised surface antigens have been proposed as markers of macrophage activation (Mackay et al, 1989). Ia (MHC class II) expression gives an overall assessment of exposure to immunogen and lymphokine. Assays of MMR differentiate resident or elicited macrophages from activated macrophages while assays of H_2O_2 generation discriminate between immunologically unstimulated (resident and elicited) and activated populations.

Insulin-like growth factor (IGF-1) has been established as an important mediator of growth hormone (GH) effects throughout post-natal life. As well as having insulin-like effects on mesenchymal cells in vitro, it is mitogenic for many different types (Daughaday and Rotwein, 1989), and is a potent adipocytic differentiation factor. IGF-1 is secreted by macrophages and a variety of other cell types.

The MMR is a lectin-like endocytic receptor which binds mannosylated glycoproteins and particles. It has a large number of possible physiologic and microbial ligands and phagocytic potential that together suggest a likely role in host defense. The MMR is a useful marker of macrophage maturation (Ezekowitz and Stahl, 1988). It is not expressed on freshly isolated human monocytes, but following in

vitro culture its expression is induced co-incident with the acquisition of macrophage endocytic and secretory functions, for example increased phagocytic activity of Fc and complement receptors, and enhanced secretion of lysosomal enzymes. MMR expression reaches a stable peak after 3-5 days in culture. Despite its potential use as a macrophage maturation marker, relatively few studies have attempted to define soluble regulators of MMR expression.

The interactive nature of biologic systems suggests that activation events are usually counterbalanced by negative regulatory mechanisms. TGF-b1 and -b2 (T-cell growth factor b1 and b2) (Tsunawaki et al, 1986) and macrophage deactivating factor (MDF) (Srimal and Nathan, 1990) inhibit macrophage ROI (reactive oxygen intermediate) generation and microbicidal activity as well as the priming effect of IFN-g and have been described as macrophage de-activators (MDAFs - used herein to refer to deactivation of microbicidal activity). MDF has been purified to near homogeneity but its relationship to other molecules, for example IL-10 (interleukin 10), is unknown, although a recent report suggests it is distinct (Bogdan et al, 1991). As a potential source of many of these so-called MDAFs, in particular TGF-b, and PGE_2 (Prostaglandin E_2) (Davies et al, 1980), macrophages themselves may have a built in negative feedback mechanism for decreasing pro-inflammatory activity. MDAFs have convincingly been shown to induce or potently enhance the release of growth promoting factors or protease inhibitors.

An established model of macrophage immunologic activation involves the notion of macrophage activation/de-activation as a linear but reversible pathway from th circulating monocyte, through inflammatory recruitment, towards maximal

microbicidal and tumoricidal activity. This traditional view has become too narrow to accommodate the functional diversity of cytokine actions, but remains useful in highlighting the adaptive capacity of macrophages to respond to micro-evnironmental signals. Further, the use of specific sets of in vitro assays as indicators of immunologic activation is helpful in defining a role for a particular set of cytokines within various models of immunity.

The ability of macrophages to penetrate areas of inflammation and to respond rapidly to local conditions is crucial to their role in host defense. The list of cytokines now described is long and growing and macrophages, at least in tissue culture, are capable of producing a large number of these. The local regulation of macrophage secretion is likely to be very important in determining the physiological role of macrophages and knowledge in this area is essential for understanding and manipulation of host defenses.

It has long been known that pathogens may evoke a predominantly cell-mediated or a largely humoral immune response. While the physicochemical properties of the different antigens, the route of administration and the dose appear to be factors controlling the nature of the subsequent immune response, cellular mechanisms controlling immune response selection are becoming clearer. Recently, T-helper subsets expressing mutually inhibitory cytokine profiles have been demonstrated in various mammalian models of infection. Within the mouse, two functionally distinct T-helper (Th) cell populations, Th-1 and Th-2 cells, have been well characterized. Th-1 cells secrete IL-2, IFN-g and lymphotoxin/TNF

(tumour necrosis factor). In contrast, Th-2 cells do not express IL-2, IFN-g or TNF but s cr te high levels of IL-4, IL-5, IL-6 and IL-10. Both subsets produce IL-3 and GM-CSF (reviewed by Mosmann and Coffman, 1991). Functionally distinct helper T-cell subsets correlate well with the development of different immune responses. For example, in mice, Th-1 cells are associated with cell-mediated immunity and DTH (delayed-type hypersensitivity) reactions, while Th-2 cells promote humoral immunity. In addition, effective host-defense against some infections appears to be dependent on the balance between Th-1 and Th-2-derived lymphokine production. For example, Leishmanial infection of susceptible Balb/c mice induces a predominantly Th-2 response, while resistant C57/BL6 animals produce a predominantly Th-1 response (Locksley et al, 1991). Th-1 cell clones derived from resistant C57/BL6 mice adoptively transferred to susceptible Balb/c mice provide protection against lethal infection with Leishmania major. Conversely, Th-2 derived cytokines exacerbate infection in C57/BL6 animals.

In humans a similar association of Th-2 cell responses with failure of effective cell-mediated killing of a pathogen has been made in patients with lepromatous leprosy (Yamamura et al, 1991). Th subsets have not been elucidated in humans to the same extent as in rodents but recent results show human T-cell clones with the lymphokine profiles of murine Th cells (Salgame et al, 1991).

The balance of cytokine production by different T-helper cells and macrophages is likely to be crucial to elimination of the pathogen and return

of the inflammatory focus to the normal state. Delayed-type hypersensitivity reactions on th one hand are impressive examples of immune imbalances leading to excessive macrophage inflammatory activity and tissue damage, while on the other hand, the anergy of lepromatous leprosy of miliary tuberculosis patients are examples of inadequate host-defense. Although the effects of IFN-g and TNF on macrophages have been well characterised, the influence of Th-2 cytokines on macrophage function is poorly understood.

IL-4 has apparently contradictory effects on macrophage host defense function, profoundly suppressing many activities, for example inflammatory cytokine release, while stimulating variable expression of MHC class 2 antigen on different macrophage populations. The definitive roles of IL-4 in regulating macrophage immune function are therefore unclear, as are those of IL-10.

In vitro studies suggest IL-10 is a potent inhibitor of macrophage dependent antigen presentation to Th-1 cells. In addition, it inhibits inflammatory cytokine production by an IL-4, TGF-b and MDF independent mechanism, inhibits IFN-g priming of the respiratory burst and suppresses Th-1 subset cytokine secretion. Therefore, it is likely to powerfully suppress delayed type hypersensitivity reactions. However, it does not inhibit Th-2 cells, stimulates MHC class 2 ag expression on B-cells and promotes cytotoxic T-cell capability.

The pr sent invention is based on the discovery that stimulation of elicited macrophages (BgPM) with Th-2 supernatant or TH-2 derived lymphokines, IL-4 and IL-10, with or without IL-5 and IL-6, results in a clearly distinguishable phenotype, herein called a healing phenotype, with dramatically altered endocytic and secretory properties in comparison with IFN-g activated macrophages. These results, obtained from in vitro assays, are used to expand the conceptual framework of macrophage immunologic activation beyond the current idea which is based on the action of single cytokines and restricted to microbicidal activation or de-activation.

In one aspect the invention provides a composition suitable for use in the treatment of an acute or chronic inflammation which comprises IL-4 and IL-10. Also provided is a composition suitable for use in the treatment of an acute or chronic inflammation which comprises Th-2 cell supernatant.

A further aspect of the invention is a composition for use in the treatment of an acute or chronic inflammation which comprises supernatant of macrophages with a healing phenotype. The macrophages may have a healing phenotype as a result of treatment with a mixture comprising Th-2 cell supernatant, and/or as a result of treatment with a mixture comprising IL-4 and Il-10.

The invention further provides a method of treatment of an acute or chronic inflammation comprising applying supernatant of macrophages with a healing phenotype to the inflammation to promote or enhance tissue repair.

Another method according to the invention is the treatment of an acute or chronic inflammation comprising applying IL-4 and IL-10 to the inflammation to promote or enhance tissue repair.

The invention also provides a method of treatment of an acute or chronic inflammation comprising applying IL-4 and IL-10 to the inflammation to promote or enhance tissue repair.

Another aspect of the invention is a method of producing macrophages with a healing phenotype in vitro by contacting a population of macrophages with a composition comprising IL-4 and IL-10, and/or Th-2 cell supernatant.

A further aspect is a method of promoting healing which comprises applying a mixture of IL-4 and IL-10 to an acute or chronic inflammation to produce macrophages with a healing phenotype.

Also provided by the invention is a method of identifying the physiological mediators of wound repair by identifying the products of macrophages with a healing phenotype and testing the products for their healing effect.

EXAMPLES

Materials and Methods

1. Th-1 and Th-2 clones

Th-1 (KLH ag specific) and Th-2 (conalbumin ag specific) clones were obtained from Dr. T. Mosmann (DNAX Research Institute, Palo Alto, Ca.). The Th-2 clone was lost after the second passage and the Th-1 clones failed to produce IFN-g following 3 weeks in culture. Th refore, stored cell sup rnatants and

subsequently combinations of recombinant proteins that correspond to the appropriate T-h cell subset were used for these studi s.

2. Animals

Mice were bred and housed at the Sir William Dunn School of Pathology, University of Oxford. In general, adult male Balb/c mice were used between age 8-12 weeks for experiments.

3. Media and reagents

Dulbecco's modified Minimum essential medium (DMEM), RPMI and OPTIMEM, a serum-free proprietary reagent, were obtained from Gibco-Biocult Ltd., Paisley, Scotland. Fetal bovine serum (FBS) was obtained from Seralab UK, Crawley Down, Sussex, England and routinely heat inactivated for 30 minutes at 56°C. Media were supplemented with 10% foetal bovine serum (FBS), glutamine (2mM), penicillin (50µg/ml) and streptomycin (100µg/ml). Phosphate-buffered saline (PBS) pH-7.4 was obtained from Oxoid Ltd. Basingstoke, U.K. LPS (E.Coli 0111:B4), zymosan A and mannan (both from Saccharomyces cerevisiae) were obtained from Sigma Chemical company, St. Louis, Mo.

The following highly purified recombinant murine proteins were gifts from the indicated source. Interferon gamma (5×10^5U/ml) from Dr. F. Balkwill, ICRF, London, U.K. IL-4 from Dr. S. Gillis, Immunex Corporation, Seattle, Ca, U.S.A. TNF alpha from Dr. E. Havell, Trudeau Institute, Saranac Lake, U.S.A. GM-CSF from Dr. M. Mielke, Freie Universiteit, Berlin, Germany. M-CSF and IL-3 from British Biotechnology, Oxford, U.K. IL-6 from Dr. Van Snick, Leuven

University, Belgium. IL-10 from Dr. Kevin Moore, DNAX Research Institute, Palo Alto, Ca., U.S.A. Human TGF beta from Dr. J. Heath, Oxford University, U.K. Human RANTES and MIP-1a from Dr. T. Schall, Genentech Inc., San Francisco, Ca., U.S.A.

4. Cells

Macrophages were isolated from the mouse peritoneal cavity. Thioglycollate broth (1ml), proteose-peptone solution (1ml of a 1% solution) and Bio-Gel (biogel) bead-elicited macrophages were isolated 4 or 5 days after intra-peritoneal injection. Biogel beads were washed by repeated centrifugation, autoclaved and ≈1 ml of a 2% v/v suspension injected ip. Cells were routinely plated at 3 x 10^5 macrophages/well in 24 well tissue culture plates. The cells were incubated for 1 hour at 37°C in a 5% CO2 incubator and then washed 4x with PBS at 4°C to remove non-adherent cells. The cells were treated within 12 hours of harvest unless otherwise indicated, when adherent monolayers consisted of >90% macrophages, and viability was >97% by phase contrast microscopy and trypan blue exclusion.

For RNA isolation, washed Bio-Gel bead-elicited peritoneal macrophages (BgPM) were incubated in 10cm bacterial plastic plates as before but left in RPMI, routinely supplemented with 10 mM glutamine, 10% FBS, penicillin and streptomycin, overnight. The cells became non-adherent and were easily washed off the dishes. These cells were centrifuged, resuspended at 10^7 cells/ml in serum free OPTIMEM and gently layered onto either a preformed ficoll step gradient or a 15% metrizamide/PBS pH7.4 solution and spun at

850g (2000 rpm in a refrigerated (4°C) Beckman benchtop centrifuge) for 15 minutes. Percoll gradients wer made with sterile percoll (Pharmacia), mixed with distilled water and 10-fold PBS to make isotonic buffers of the required densities. Each 1 ml fraction was carefully layered into a 10 ml conical centrifuge tube to make stepped gradients of the following densities (g/ml): (1) 1.100, (2) 1.090, (3) 1.080, (4) 1.075, (5) 1.070, (6) 1.060. The macrophage fraction was present at the interface of fractions 5 and 6 (ficoll method) or at the metrizamide/medium interface (metrizamide method). The macrophage fraction (>99.5% pure by immunocytochemistry (F4/80 and FA.11 ag analysis), phase microscopy and latex ingestion) was collected and re-plated before cytokine treatment. RNA was isolated as described later.

5. <u>TNF bioassay</u>

TNF levels in macrophage culture supernatants were measured using a modified mouse L929 fibroblast toxicity assay as previously described by Flick and Gifford, 1984. L929 cells were routinely tested and shown to be free of mycoplasma. Actinomycin D (1µg/ml) treated cells were incubated with test samples for 18 hours. The cells were washed, fixed in methanol and stained with crystal violet (1% w/v)/methanol. Changes in absorbance at 510 nm were quantitated by a Dynatech MR600 microtiter plate reader (Dynatech Laboratories, Alexandria, VA, USA) and converted to units per millilitre based on a standard curve using murine rTNF. To compare TNF release by different Mø populations, 3×10^5 Mø were

plated in each well and cell protein determined as required. A blocking anti-TNF antibody was routin ly used with each sample to ensure that all L929 cell death was due to TNF.

6. Macrophage mannosyl receptor (MMR) assays

These assays were performed as described previously (Stahl et al, 1980) with modifications as indicated. Briefly, mannosylated-BSA (E-Y Laboratories, 127N Amphlett Blvd., San Mateo, CA.) was trace labelled with Na(125)I by a modified chloramine-T method. Ligand was tested for trichloroacetic acid (TCA) precipitability before use.

Binding of mannose-specific ligands was assayed at saturating concentrations of ligand using trace labelled mannose-BSA (saturation 250 ng/ml ligand/5 x 10^5 Mø) in the presence or absence of mannan (5 mg/ml) or 100 fold excess unlabelled mannose-BSA. Binding was assayed after one hour at $4^{o}C$. Cells were washed in ice-cold PBS with 10 mM sodium azide. Then 500 µl of 1 M NaOH was added to dissolve the cells and the cell-associated radioactivity measured in a Packard gamma spectrometer (Packard Instrument Co. Inc., Downes Grove, IL, U.S.A.). Results were expressed as nanograms of mannose-BSA specifically bound or taken up per 5 x 10^5 Mø plated.

Degradation of ^{125}I-mannose-BSA by Mø was measured by the appearance of TCA-soluble labelled material in the culture medium. Degradation of ^{125}I-mannose-BSA is detectable after ≈40 minutes incubation at $37^{o}C$ and continues at a linear rate for several days if Mø are maintained in the continuous presence of

ligand. Trace amounts of sterile ligand (≈10^6 C.P.M. in 10 µl) were added to monolayers of adher nt Mø populations. Cells were incubated for 16 hours (unless otherwise indicated) and a 0.4 ml aliquot of medium removed to microfuge tubes. Trichloroacetic acid was added to a final concentration of 10% w/v, the tubes incubated on ice for 30 minutes and then spun for 10 minutes in a centrifuge. Supernatant (0.2 ml) was removed and 5µl of potassium iodide (4M) followed by 10µl of H_2O_2 were added to each aliquot, incubated for 10 minutes at room temperature, followed by the addition of 0.8 ml of chloroform. The mixture was vortexed vigorously, spun and 100 µl of the clear aqueous phase assayed in a gamma counter. Cell-dependent, saturable degradation of ^{125}I-mannose-BSA per unit time was calculated as a function of Mø number. Cell-free blanks were used routinely.

7. Superoxide release assay

Superoxide anion release assay measures the change in colour of cytochrome C when reduced by superoxide anion released from the stimulated macrophage. The assay described below is based on the assay described by Johnston, R.B. et al, 1978. Macrophages were plated at 1-3 x 10^5/well in RPMI 1640, 10% FCS or OPTIMEM (as indicated in figure legends) in a 24 well tissue culture plate. The density ensures that oxygen anion release is proportional to cell number. Monolayers were washed in Hanks balanced salt solution (HBSS; Gibco) without phenol red. The cells were pre-incubated for 5 minutes in a reaction mixture containing:

- Hanks buffered salin solution (HBSS)
- 80 µM Ferricytochrome C (Sigma Chemical Co., type IV)
- 2mM Sodium azide
- 10 mM Sodium phosphate buffer, pH7.4
- 10 mM glucose

Negative controls contained bovine superoxide dismutase (Sigma Chemical Co., type I) final concentration of 30µg/ml. After 5 minutes, stimuli were added for 60 minutes. Stimuli: Phorbol myristate acetate (PMA) (Sigma Chemical Co.) was added to a final concentration of 20 - 100 ng/ml. PMA was maintained at -70°C in a stock solution of 2mg/ml in dimethylsulpoxide (DMSO) (Siga Chemical Co.). DMSO alone did not stimulate cytochrome C reduction by cultured cells. In some cases zymosan (32-64µg/ml) was used as an additional stimulus. Thereafter, the plates were placed on ice, an aliquot of the reaction mixture diluted three fold with cold HBSS, centrifuged at 1500 rpm for 5 minutes, and the resulting colour change measured by spectrophotometric analysis. Superoxide release was calculated from the difference in O.D. at 550 nm, in the absence and presence of superoxide dismutase according to the formula:

$$\frac{\text{Difference in O.D. 550} \times 3 \times 0.5}{0.0211}$$

where the difference in O.D. is the sample minus the superoxide inhibitable value, 3 is the dilution factor, 0.5 the volume of the reaction mixture and 0.0211 the extinction coefficient of ferricytochrome C at 550nm. Each reaction was run in duplicate or triplicate as indicated. A cell blank was routinely included as an additional control.

8. Lysozyme assay

Lysozyme activity was measured using the "lysoplate" assay described by Osserman and Lawlor (1966). Micrococcus lysodeikticus cell walls (Sigma Chemical Co.) were suspended at 0.3mg/ml in 1% molten agarose in 10mM Tris-HC1 pH 7.0, and poured into 10 cm diameter petri dishes. Wells were punched into the set agar plates, and lysozyme containing samples placed in the wells (20µl per well). A clear zone of lysis around the well, appearing after 4-8 hours, indicated lysozyme activity. For quantisation, the radius of the zone of lysis was measured, and a standard curve constructed showing radius of lysis/hr versus µg of hen egg lysozyme (Sigma Chemical Co.), in the range 0.03µg to 10µg.

General molecular biology techniques used were based on ones described in detail in a standard laboratory manual. Western blotting for TNF was based on the procedure described by Burnette (1981).

EXAMPLE 1

Immunologic activation by Th-1 and Th-2 lymphokine: regulation of MMR activity. (see figure 1)

BgPM were harvested, plated (3×10^5 macrophages per well) and washed prior to co-incubation with Th-1 or Th-2 lymphocyte clone conditioned medium or combinations of IFN-g (50U/ml) and TNF (500U/ml), or IL-4 (1ng/ml) and IL-10 (100U/ml) as indicated. Dexamethasone (DEX) was used at 10^{-7}M. Monolayers were incubated for 48 hours prior to addition of ^{125}I-mannose-BSA (6×10^6 cpm/µg) with or without excess

unlabelled mannose-BSA. Following overnight incubation, supernatants were removed for degradation analysis as in Materials and Methods. Where sequential addition of lymphokines was necessary (IL>IFN-g: IFN-g->IL-4), monolayers were incubated in the presence of the primary stimulus, washed, and the secondary stimulus added for the following 24 hours prior to the addition of ligand. Results are displayed in figure 1 and show mean +/- SD of pooled results of three independent experiments.

EXAMPLE 2

Th-lymphokine stimulated BgPM gene expression: RT-PCR analysis. (see figure 2)

Expression of TNF, MMR, IGF-1 and lysozyme mRNA transcripts by BgPM purified by differential density centrifugation 1.5 x 10^5 macrophages (99.8% pure by F4/80 ag immunocytochemistry) were plated in OPTIMEM in 24 well plates, and incubated in the presence or absence of either IFN-g (50U/ml) and TNF (500U/ml), or IL-4 (1ng/ml) and IL-10 (100U/ml) for 48 - 56 hours. Control monolayers were mock-treated with PBS. Separate sets of monolayers (right hand side of the figure) were further stimulated with LPS (1ug/ml) for 2 hours. Thereafter, the monolayers were washed and lysed in a GuSCN solution. Total RNA was isolated, reverse transcribed and cDNA fragments were amplified by PCR. PCR products were subjected to 1% AGE, the gels stained with ethidium bromide and photographed. Size markers (123 bp ladder) were used to estimate fragment sizes. PCR bands were subjected to restriction enzyme digestion to confirm the

identity of the amplified products except in the case of IGF-1 products, which reveal characteristic double bands corresponding to IGF-1 type A and B. Experiments were performed at least three times using independent cell preparations. Each lane represents a separately processed well. Arrows indicate expected fragment sizes.

A: TNF, MMR and lysozyme (LYZ). B: IGF-1 (IGF) and lysozyme.

LHS		RHS
1 = control	- duplicate lanes -	4 = control + LPS
2 = IL-4/IL-10	-triplicates lanes-	5 = IL-4/IL-10 + LPS
3 = IFN-g/TNF	-triplicates lanes-	6 = IFN-g/TNF + LPS

A and B represent independent experiments. Input cDNA was representative of about 50 cell equivalents per lane except for lysozyme PCR products shown in B (bottom panel), where input cDNA was representative of 20 cell equivalents. Photographic exposure times were not constant, as may be assessed by the difference in intensity of the various marker lanes.

EXAMPLE 3 - (see figure 3(a) and (b)).

(a) IL-1 gene expression

Separate experiment comparing IL-1, MMR and IGF-1 gene expression, conducted as described in the legend to figure 2 except indicated monolayers were incubated in the presence of LPS for 1.5 hours. cDNA corresponding to equal numbers of macrophages, treat d as indicat d, w re analysed by PCR, 1% AGE and ethidium bromide staining and photography.

Top panel: IL-1

LHS		RHS
1 = control	- duplicates -	4 = control + LPS
2 = IL-4/IL-10	-triplicates -	5 = IL-4/IL-10 + LPS
3 = IFN-g/TNF	-triplicates -	6 = IFN-g/TNF + LPS

Bottom panel: MMR and IGF-1 expression, in the absence and presence of LPS, respectively, were assayed in parallel and are shown for reference.

(b) MCP-1 gene expression

Adherent BgPM express MCP-1 transcripts. BgPM were incubated in OPTIMEM in the presence of either (1) IL-4/IL-10, (2) IFN-g (50U/ml) alone or (3) PBS control. Following overnight (16 hours) incubation, total RNA was isolated and reverse transcribed. cDNAs representing (1) 1000, (b) 100 and (c) 10 cell equivalents were subjected to PCR. The resulting products were analysed by 1% AGE, ethidium bromide staining and photography as before.

1 = IL-4/IL-10
2 = IFN-g
3 = control

EXAMPLE 4

Comparison of the effect of Th-1 and Th-2 lymphokine on
BgPM superoxide secretion, TNF release and Ia expression. (see figure 4)

Adherent BgPM (3 x 10^5 per well) were incubated for 56 hours in the presence of: IL-4 (1ng/ml)(+) IL-4 + IL-10, IFN-g, or medium (OPTIMEM) alone(o). Except for IL-4 (alone)(+) and medium control (o), indicated as single data points, lymphokines were used at 10-fold dilutions with starting concentrations as follows: IL-4 (1ng/ml) and IL-10 (50U/ml); IFN-g (100U/ml). LPS (1µg/ml), PMA (20ng/ml) or zymosan (64 µg/ml) were used as secondary triggers as indicated, except for IL-4 alone or medium control, where zymosan was used as a secondary trigger. Top panel: TNF release was measured by L929 bioassay, following 16 hours LPS or zymosan stimulation. Middle panel: O_2 release assayed 1 hour following PMA (100ng/ml) or zymosan (64µg/ml stimulation. Superoxide dismutase (25µg/ml) inhibited all O_2 reduction of ferricytochrome C. Results are expressed as nmol O_2/mg cell protein/60 minutes. Bottom panel: Ia surface expression was assayed by saturation indirect-binding assays using mAb, TIB120 anti-H-2 (Ia), and expressed as molecules per cell. Results shown are representative of pooled results of two independent experiments, except for IL-4 + IL-10 incubations where data are representative of one experiment done in triplicate.

EXAMPLE 5

In vivo Experiments

The effect of Th-2 macrophage conditioned medium on inflammatory sites was tested in mice. Nude mice were shaved on the back and a 2cm long scratch incision was made on each. Two groups were treated at the site of the incision with the following:

1) PBS - Control
2) Th-2 macrophage conditioned medium.

The liquids were applied in a gauze swob and left for 24 hours, after which time the wounds were observed. The swobs were replaced and the wounds were observed again at 72 hours.

RESULTS

MMR activity:

Experiments using T-cell clone supernatants for their effect on MMR expression, reflected by assays of degradation of ^{125}I-mannose-BSA, indicated the contrasting effects of Th-2 derived supernatants (increased MMR) and Th-1 derived supernatants (decreased MMR) (figure 1). Similar results were obtained with combinations of recombinant IL-4/IL-10 and IFN-g/TNF. IL-4 (1ng/ml) alone induces high level MMR activity. IL-10 (5OU/ml) further enhanced MMR expression, but like dexamethasone the effect was additive rather than synergistic. Blocking mAb against IL-10, SEC-1, completely abolished the effects of IL-10. IL-5 and IL-6, alone or in combination with

IL-4, had no apparent effect on MMR expression (not shown). With respect to Th-1 lymphokine, only IFN-g had a directly inhibitory effect on MMR expression, although TNF blocked IL-4 dependent enhancement. Figure 1 demonstrates the cross-regulatory influence of Th-1 and Th-2 derived cytokines in which IFN-g or TNF completely inhibit the effect of IL-4 on MMR activity. In the presence of saturating doses of both IL-4 and IFN-g, no enhancement of MMR activity occurs. However, if IFN-g is added more than 24 hours after IL-4 it does not fully reverse the enhanced MMR expression within the following 48 hour period. MMR activity of secondarily IFN-g treated BgPM was 84% of the MMR levels of control cells (IL-4 treated, no secondary stimulus). Of the other cytokines or growth factors tested, only M-CSF moderately (2-fold) enhanced MMR expression. IL-3, produced by both Th-1 and Th-2 cells, induced cell division, but did not increase MMR expression when expression was corrected for cell number (not shown).

Inflammatory cytokine and IGF-1 gene expression:

The effect of IL-4 and IL-10 on macrophage gene expression was compared with effect on IFN-g and TNF. cDNAs from the different macrophage populations were prepared from equal cell numbers cultured in the same 24 well plate. Therefore, differences in specific gene expression in the various conditions are directly comparable. Easily detectable differences in the PCR reaction products reflect significant variation in mRNA expression. However, it is not possible to compare mRNA levels of different molecules. Figures 2 and 3 show that IL-4/IL-10

partially suppresses IL-1 and TNF mRNA levels, while IGF-1 is highly expressed. In direct contrast, IFN-g/TNF enhances IL-1 (fig. 3) and TNF (fig. 2), but suppresses IGF-1 expression. Lysozyme gene expression is unaffected by either treatment. There is a minimum 16-fold difference between the IL-4/IL-10 and the IFN-g/TNF groups with respect to IGF-1 and MMR expression as measured by titrations of input cDNA (not shown). MCP-1 gene expression was similarly inhibited by IL-4/IL-10 and enhanced by IFN-g/TNF (fig. 3 - bottom panel). LPS induced TNF and IL-1 mRNA to an apparently similar extent in control cells, IL-4/IL-10 and IFN-g/TNF treated groups. Bioassay for TNF release following LPS (1 µg/ml) stimulation confirmed the TNF mRNA data, at the protein level (fig. 4 - top panel). In addition, samples of Th-2 macrophage conditioned supernatants were freeze dried, and IGF-1 radio-immunoassay (RIA) performed. Preliminary results indicate a dramatic increase in IGF-1 immunoreactivity in LPS stimulated Th-2 macrophage conditioned medium (data not shown).

TNF release and respiratory burst activity:

Results of respiratory burst analysis of the two populations are compared with the effect on TNF release and Ia expression (fig. 4). IL-4/IL-10 suppresses PMA triggered O_2 release. IL-4 alone has no effect on PMA triggered respiratory burst (not shown), but apparently enhances zymosan triggered O_2 release. IFN-g primes BgPM for subsequent phagocytic or PMA induced O_2 release, as previously shown (Nathan et al, 1983). IFN-g in combination with IL-10 failed to prime cells for subsequent PMA trigger of release

(not shown). IL-4/IL-10 was inhibitory for PMA triggered O_2 release, but had less effect on zymosan induced release. Similarly, TNF release and Ia expression are inhibited by IL-4/IL-10 treatment and enhanced by IFN-g stimulation. TNF secretion following zymosan challenge was only partially suppressed by IL-10/IL-4 stimulation.

Ia expression:

Indirect-binding assays (fig. 4) demonstrate the inhibition of Ia expression by IL-4/IL-10 in comparison to its enhancement by IFN-g.

EXAMPLE 5

In vivo Experiment

After 24 hours there was no noticeable difference between the wound sites of the controls and treated mice. At 72 hours however, there was evidence of markedly better healing in the group treated with Th-2 macrophage conditioned medium.

Discussion

MMR activity:

The effect of IL-10, alone and in combination with IL-4, on MMR expression has been confirmed at the protein and mRNA levels. The strikingly enhanced activity of murine MMR, a phagocytic receptor associated with host-defense function, is indicative of alternative macrophage immunologic activation by Th-2 derived cytokines.

Inflammatory cytokine and IGF-1 gene expression:

IFN-g/TNF treatment, in the absence of a further trigger, enhances expression of IL-1 and TNF expression. However, following LPS challenge, IL-4/IL-10 treatment did not measurably inhibit TNF or IL-1 mRNA levels of TNF or IL-1. At the protein level, IL-4/IL-10 treatment clearly blocks release of bioactive TNF; and northern blots (results not shown) indicate that both murine and human IL-4, and human IL-10, inhibit expression of TNF following LPS challenge. PCR is probably too sensitive to detect the relatively smaller differences in expression of IL-1 and TNF as compared to IGF-1 and MMR. In addition, TNF mRNA levels are post transcriptionally regulated (Beutler et al, 1986) and it would not have been surprising to find an apparent discrepancy between mRNA levels and protein secreted.

Respiratory burst modulation:

The increased O_2 release from zymosan stimulated IL-4 treated BgPM is probably the result of greatly enhanced ligation of MMR. Therefore, it may not reflect priming in the same sense as that observed with IFN-g.

Ia expression:

Data presented in fig. 4 and 5, show that IL-4/IL-10 inhibits Ia expression on elicited mouse macrophages.

Tak n together, thes data indicat that Th-1 activated BgPM (low MMR and IGF-1; high Ia and TNF)

may be easily distinguished from Th-2 activat d macrophages (high MMR and IGF-1, low Ia and TNF). In addition, the data suggest that Th-2 derived cytokines provide an alternative pathway for macrophage activation as compared to the immunologic activation that occurs during infections in which IFN-g activates macrophages.

An extended model of macrophage immunologic activation is presented in fig. 5, in which 1 represents Enhanced microbicidal activity against facultative i/c pathogens; 2 represents Immunologic activation; 3 represents possible specific microbicidal function, and repair functions. The model illustrates the possible influence of soluble and/or surface interactions of Th-1 and Th-2 lymphocytes on macrophage function within inflammatory infiltrates. Th-1 and Th-2 lymphokine may act sequentially or simultaneously on macrophage function, therefore macrophage deactivation is not necessarily a step-wise phenomenon as implied in the previous model. Although IL-10 may be a powerful inhibitor of Ia expression and PMA triggered O_2 generation, and by extension an inhibitor of killing of some intracellular pathogens, Th-2 activated macrophages may have as yet undiscovered killing function for specific pathogens.

Th-1 and Th-2 macrophage phenotypes: in vivo correlates and functions

Th-1 macrophages are phenotypically equivalent to BCG- or IFN-g activated macrophages and probably have a role in cell-mediated immune responses and DTH reactions.

Th-2 macrophages recruited to the inflammatory site may acquire maximal and specialised host-defense function against specific pathogens, for

example mannosylated microorganisms, or parasites which evoke an IgE humoral response. Equally, the Th-2 macrophage may be used as a potent scavenger cell for the clearance of damaged cells bearing exposed mannose residues, or other as yet uncharacterised carbohydrate ligands for the MMR or CD 23. In addition, Th-2 macrophages may further promote wound healing by inhibiting the release of inflammatory stimuli and enhancing release of IGF-1, and probably other growth factors, which act on stromal cells within the local environment to promote their growth and secretion of matrix components.

Elicited macrophages may therefore be differentially stimulated to alter their function according to signals derived from different Th cell subsets. In vitro, Th-1 and Th-2 macrophage phenotypes characterised here are easily distinguished by differential expression of endocytic and secretory markers and may represent extremes or only two parts of the elcitied macrophage immunologic modulation spectrum in vivo (fig. 5 - dashed lines indicate hypothetical pathways of feedback regulation of Th cellfunction). This heterogeneity in macrophage immunologic activation emphasizes the unique host defense potential of the MPS.

REFERENCES

Beutler, B.; Cerami, A. The biology of cachectin/TNF a primary mediator of the host response. Annu Rev Immunol. 1989; 7: 625-55.

Blanden, R.V.; Hapel, A.J.; Doherty, P.C.; Zinkernagel, R.M. Lymphocyte-macrophage interactions and macrophage activation in the expression of antimicrobial immunity in vivo. In: Immunobiology of the macrophage (ed. D.S. Nelson). Academic Press, Inc., New York. Chapter 15, pp 367-395.

Bogdan, C.; Stenger, S.; Rollinghoff, M.; Solbach, W. Cytokine interactions in experimental cutaneous leishmaniasis. Interleukin 4 synergizes with interferon gamma to activate murine macrophages for killing of Leishmania major amastigotes. Eur J Immunol. 1991 Feb; 21(2): 327-33. (a)

Boraschi, D.; Censini, S.; Bartalini, M.; Scapigliati, G.; Barbarulli, G.; Vicenzi, E.; Donati, MB; Tagliabue, A. Interferon inhibits prostaglandin biosynthesis in macrophages; effects on arachidonic acid metabolism. J Immunol. 1984 Apr; 132(4): 1987-92. (a)

Boraschi, D.; Censini, S.; Tagliabue, A. Interferon gamma reduces macrophage suppressive activity by inhibiting prostaglandin E2 release and inducing interleukin 1 production. J Immunol. 1984 Aug; 133(2) : 764-8. (b)

Coffman, RL; Mosmann, TR. CD4+ T cell subsets; regulation of differentiation and function. Res Immunol. 1991 Jan; 142(1): 7-9.

Davies, P.; Bonney, RJ; Humes, JL; Kuehl, FA. The role of macrophage secretory products in chronic inflammatory processes. J Invest Dermatol. 1980 May; 74(5): 292-6.

Daughaday, WH; Rotwein, P. Insulin like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. Endocr Rev. 1989 Feb; 10(1): 68-91.

Ezekowitz, RA; Stahl, PD. The structure and function of vertebrate mannose lectin like proteins. J Cell Sci. Suppl. 1988; 9: 121-33.

Locksley, RM; Heinzel, FP; Holaday, BJ; Mutha, SS; Reiner, SL; Sadick, MD. Induction of Th1 and Th2 CD4+ subsets during murine Leishmania major infection. Res Immunol. 1991 Jan; 142(1): 28-32.

MacKay, RJ; Pace, JL; Jarpe, MA; Russell, SW; Macrophage activation associated proteins. Characterization of stimuli and conditions needed for expression of proteins 47b, 71/73, and 120. J Immunol. 1989 Mar 1.; 142: 1639-45.

Murray, HW; Byrne, GI; Rothermel, CD; Cartelli, DM. Lymphokine enhances oxygen independent activity against intracellular pathogens. J Exp Med. 1983 Jul 1; 158(1): 234-9. (a)

Murray, HW; Cartelli, DM. Killing of intracellular Leishmania donovani by human mononuclear phagocytes. Evidence for oxygen dependent and independent leishmanicidal activity. J Clin Invest. 1983 Jul; 72(1): 32-44. (b)

Nathan, CF; Murray, HW; Wiebe, ME; Rubin, BY. Identification of interferon gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. J Exp Med. 1983 Sep 1; 158(3): 670-89.

Reed, SG; Nathan, CF; Pihl, DL; Rodricks, P.; Shanebeck, K.; Conlon, PJ; Grabstein, K. H. Recombinant granulocyte/macrophage colony stimulating factor activates macrophages to inhibit Trypanosoma cruzi and release hydrogen peroxide. Comparison with interferon gamma. J Exp Med. 1987 Dec 1; 166 (6): 1734-46.

Salgame, P; Abrams, JS; Clayberger, C; Goldstein, H; Convit, J; Modlin, RL; Bloom, BR. Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. Science 1991. 254: 279-282.

Schreiber, RD: Celada, A.; Buchmeier, N. The role of interferon gamma in the induction of activated macrophages. Ann Inst Pateur Immunol. 1986 Mar Apr; 137C(2): 203-6.

Simon, HB; Sheagren, JN. Enhancement of macrophage bactericidal capacity by antigenically stimulated immune lymphocytes. Cell Immunol. 1972 Jun; 4(2): 163-74.

Srimal, S.; Nathan, C. Purification of macrophage deactivating factor. J exp Med. 1990 Apr 1; 171(4): 1347-61.

Tsunawaki, S.; Nathan, CF. Macrophage deactivation. Altered kinetic properties of superoxide producing enzyme after exposure to tumor cell conditioned medium. J Exp Med. 1986 Oct 1; 164(4): 1319-31.

Warren, MK; Vogel, SN. Opposing effects of glucocorticoids on interferon gamma induced murine macrophage Fc receptor and Ia antigen expression. J Immunol. 1985 Apr; 134(4): 2462-9.

Yamamura, M; Ukemura, K; Deans, RJ; Weinberg, K; Rea, TH; Bloom, BR; Modlin, RL. Defining Protective responses to pathogens: cytokine profiles in leprosy lesions. Science 1991. 254: 277-279.

CLAIMS

1. A composition suitable for use in the treatment of an acute or chronic inflammation which comprises IL-4 and IL-10.

2. A composition suitable for use in the treatment of an acute or chronic inflammation which comprises Th-2 cell supernatant.

3. A composition for use in the treatment of an acute or chronic inflammation which comprises supernatant of macrophages with a healing phenotype.

4. The composition of claim 3, wherein the macrophages have a healing phenotype as a result of treatment with a mixture comprising Th-2 cell supernatant.

5. The composition of claim 3, wherein the macrophages have a healing phenotype as a result of treatment with a mixture comprising IL-4 and IL-10.

6. A method of treatment of an acute or chronic inflammation comprising applying supernatant of macrophages with a healing phenotype to the inflammation to promote or enhance tissue repair.

7. A method of treatment of an acute or chronic inflammation comprising applying IL-4 and IL-10 to the inflammation to promote or enhance tissue repair.

8. A method of producing macrophages with a healing phenotype in vitro by contacting a population of macrophages with a composition comprising IL-4 and IL-10, and/or Th-2 cell supernatant.

9. A method of promoting healing which comprises applying a mixture of IL-4 and IL-10 to an acute or chronic inflammation to produce macrophages with a healing phenotype.

10. A method of identifying the physiological mediators of wound repair by identifying the products of macrophages with a healing phenotype and testing the products for their healing effect.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 93/00719

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K37/02; C12N5/08; C12Q1/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K ; C07K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	JOURNAL OF IMMUNOLOGY. vol. 147, no. 11, 1 December 1991, BALTIMORE US pages 3815 - 3822 D.F. FIORENTINO ET AL 'IL-10 inhibits cytokine production by activated macrophages' see the whole document especially page 3816 and the discussion. ---	1-10
A	JOURNAL OF IMMUNOLOGY. vol. 140, no. 5, 1 March 1988, BALTIMORE US pages 1548 - 1554 A. A. TE VELDE ET AL 'Modulation of phenotypic and functional properties of human peripheral blood monocytes by IL-4' see the whole document ---	1-10

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
23 JULY 1993	12. 08. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	LE CORNEC N.D.R.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,A	JOURNAL OF EXPERIMENTAL MEDICINE vol. 176, July 1992, pages 287 - 292 M. STEIN ET AL 'Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation' * see the whole document especially page 287 lines 18-24 and the discussion * ---	1-10
A	JOURNAL OF IMMUNOLOGY. vol. 148, no. 6, 15 March 1992, BALTIMORE US pages 1792 - 1796 R. T. GAZZINELLI ET AL 'IL-10 inhibits parasite killing and nitrogen oxide production by IFN-gamma-activated macrophages' see page 1795, line 24 - line 40 see abstract ---	1-10
A	JOURNAL OF EXPERIMENTAL MEDICINE vol. 174, November 1991, pages 1209 - 1220 R. DE WAAL MALEFYT ET AL 'Interleukin 10 inhibits Cytokine synthesis by human monocytes: an autoregulatory role of Il-10 produced by monocytes' see abstract see the discussion see page 1215, line 14 - line 17 ---	1-10
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 86, May 1989, WASHINGTON US pages 3803 - 3807 P. H. HART ET AL 'Potential antiinflammatory effects of Interleukin 4: Suppression of human monocyte tumor necrosis factor alpha,IL-1 and PGE2' see abstract ---	1-10

-/--

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	CHEMICAL ABSTRACTS, vol. 109, no. 15, 10 October 1988, Columbus, Ohio, US; abstract no. 126810b, D. A. RAPPOLEE ET AL 'Wound macrophages express tgf-alpha and other growth factors in vivo' page 486 ;column R ; see abstract & SCIENCE vol. 241, 1988, LANCASTER, PA US pages 708 - 712	10

INTERNATIONAL SEARCH REPORT

PCT/GB93/00719

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 6-7, 9 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.